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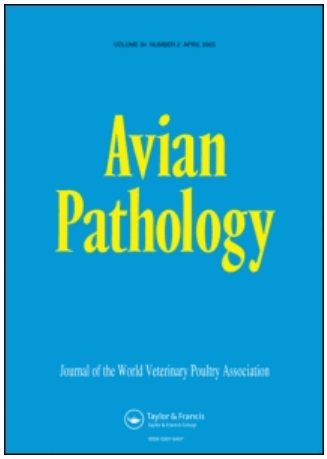

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Distribution of viral antigen gp85 and provirus in various tissues from commercial meat-type and experimental White Leghorn Line 0 chickens with different subgroup J avian leukosis virus infection profiles

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Distribution of viral antigen gp85 and provirus in various tissues from commercial meat-type and experimental White Leghorn Line 0 chickens with different subgroup J avian leukosis virus infection profiles

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Immunohistochemistry and polymerase chain reaction (PCR) were used to test for the presence of avian leukosis virus (ALV) J viral antigen gp85 and proviral DNA, respectively, in various tissues (adrenal gland, bone marrow, gonad, heart, kidney, liver, lung, pancreas, proventriculus, sciatic nerve, spleen, and thymus). Tissues were collected from 32-week-old commercial meat-type and Avian Disease and Oncology Laboratory experimental White Leghorn Line 0 chickens with the following different infection profiles: tV+A−, included *in ovo*-tolerized viraemic chickens with no neutralizing antibodies (NAbs) on any sampling; ntV+A−, included chickens that were viraemic and NAb-negative at the time of termination at 32 weeks post hatch, but had NAbs on up to two occasions; V+A+, included chickens that were viraemic and NAb-positive at the time of termination at 32 weeks post hatch, and had NAbs on more than two occasions; V−A+, included chickens that were negative for viraemia and NAb-positive at the time of termination at 32 weeks post hatch, and had antibody on more than two occasions; V−A−, included chickens that were never exposed to ALV J virus. There was a direct correlation between viraemia and tissue distribution of gp85, regardless of the NAb status and strain of chickens, as expression of ALV J gp85 was noted in only viraemic chickens (tV+A−, ntV+A−, V+A+), but not in non-viraemic seroconverted chickens (V−A+). Of the four oligonucleotide primers pairs used in PCR to identify ALV J provirus, only one primer set termed H5/H7 was useful in demonstrating ALV J proviral DNA in the majority of the tissues tested from non-viraemic, antibody-positive chickens (V−A+). The results suggest that PCR using primer pair H5/H7 is more sensitive than immunohistochemistry in identifying ALV J in chickens that have been exposed to virus, but are not actively viraemic.

Introduction

Chickens infected *in ovo* with avian leukosis viruses (ALV) develop tolerant viraemia and fail to develop neutralizing antibodies (NAbs) against the inoculated virus. However, when infected at hatch or early in life, they usually develop efficient NAbs that eliminate viraemia for prolonged periods of time. A characteristic feature of ALV J infection in meat-type chickens infected at hatch or early in life is induction-persistent viraemia even in the presence of NAbs against the inoculated strain of virus (V+A+) (Witter *et al.*, 2000; Pandiri, 2005; Pandiri *et al.*, 2007). This has also been demonstrated in other ALV subgroups and hosts. Geryk *et al.* (1996) demonstrated the reappearance of viraemia in ducks infected with ALV C in the presence of NAb. This situation is similar to bonafide persistent viruses such as lymphocytic choriomeningitis virus, human immunodeficiency virus, hepatitis B and C, foot and mouth disease virus, and other RNA viruses (Ahmed *et al.*, 1997).

Several studies on ALV tissue tropism were conducted to demonstrate the presence of virus in various tissues of the chicken using electron microscopy (Dougherty & Di Stefano, 1967; Di Stefano & Dougherty, 1969; Di Stefano *et al.*, 1973; Gilka & Spencer, 1985), immunohistochemistry (IHC) (Dougherty *et al.*, 1972; Gilka & Spencer, 1984; Arshad *et al.*, 1997; Gharaibeh *et al.*, 2001; Williams *et al.*, 2004) and molecular methods (Robinson *et al.*, 1993; Arshad *et al.*, 1999; Stedman *et al.*, 2001). All of these previous studies demonstrated ALV protein or nucleic acid in chickens that were inoculated with ALV either *in ovo* or on day of hatch;

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in most cases, sampling coincided with the viraemic phase of the infection. Distribution of ALV antigen or proviral DNA in non-viraemic seroconverted chickens is poorly understood. Arshad *et al.* (1999) examined one non-viraemic seroconverted chicken and did not detect ALV J transcripts using *in-situ* hydridization. Understanding ALV J persistence in non-viraemic seroconverted chickens is very important since recent work in our laboratory demonstrated that adult seroconverted male chickens free of viraemia and cloacal shedding for a prolonged period of time reverted to viraemia and cloacal shedding when subjected to adrenocorticotropin-induced stress (Pandiri, 2005). Hence, the objective of this study was to test for the presence of ALV J *env* antigen gp85 and provirus in various tissues (adrenal gland, bone marrow, gonad, heart, kidney, liver, lung, pancreas, proventriculus, sciatic nerve, spleen, and thymus) from chickens with various infection profiles. The different infection profiles are based on viraemia as tested by virus isolation and on NAbs as tested by virus microneutralization assay on samples collected at 8, 12, 16, 20, 24 and 32 weeks post hatch. The different infection profiles are defined as follows: tV+A−, included *in ovo*-tolerized viraemic chickens with no NAbs on any sampling; ntV+A−, included chickens that were viraemic and NAb-negative at the time of termination at 32 weeks post hatch, but had NAbs on up to two occasions; V+A+, included chickens that were viraemic and NAb-positive at the time of termination at 32 weeks post hatch, and had NAbs on more than two occasions; V−A+, included chickens that were negative for viraemia and NAb-positive at the time of termination at 32 weeks post hatch, and had antibody on more than two occasions; V−A−, included chickens that were never exposed to ALV J virus.

Materials and Methods

Samples. The samples in this study were selected retrospectively from a previous experiment involving 75 commercial meat-type chickens and 75 Avian Disease and Oncology Laboratory (ADOL) experimental White Leghorn Line 0 chickens (Crittenden & Fadly, 1985). These meat-type and ADOL Line 0 chickens were inoculated at hatch with ADOL R5-4 at 10 000 median tissue culture infectious dose by intra-abdominal route or were contact exposed at hatch. The tissue samples were selected based on the ALV J infection profile of the chickens at the end of the experiment at 32 weeks post hatch. The different infection profiles based on viraemia as tested by virus isolation and on NAb as tested by virus microneutralization assay are as defined above.

Unfortunately, this experiment did not include any tolerized chickens; that is, persistently viraemic chickens tolerized *in ovo* with no neutralizing antibody on any sampling (tV+A−). In order to serve as a positive control for the assays, one of the five ADOL Line 0 chickens that were tolerized *in ovo* (10 000 median tissue culture infectious dose by yolk sac route) to ADOL Hc1 was selected from a different experiment. The number of chickens selected for this study belonging to different infection profiles is included in Table 1.

Tissues collected for IHC and polymerase chain reaction (PCR) assays included the adrenal gland, bone marrow, gonad, heart, kidney, liver, lung, pancreas, proventriculus, sciatic nerve, spleen, thymus and tumours (if present). Tissues were embedded in Tissue-Tek® O.C.T compound (Sakura Finetek USA, Inc., Torrance, California, USA) and snap frozen in liquid nitrogen for IHC studies. Genomic DNA was isolated from all of the above tissues for PCR.

Chickens. Commercial meat-type chickens and ADOL experimental White Leghorn Line 0 chickens (Crittenden & Fadly, 1985) were used in this study. The meat-type chickens were provided by a major broiler breeder company that certified the dams were negative for ALV infection. In addition, we confirmed the ALV-negative status of the chicks at hatch by serology and virus isolation from the meconium. The chickens were housed in floor pens maintained as isolation units under biosecurity level-2 containment for 32 weeks. ADOL Line 0 chickens were provided feed and water *ad libitum*, but feed was restricted for commercial meat-type chickens to limit excessive body weight gain, as recommended by the breeder. All chickens were cared for and handled according to ADOL animal care and use committee guidelines.

Viruses. Strain ADOL Hc1, the US prototype of ALV J (Fadly & Smith, 1999) and a molecularly cloned ALV J ADOL R5-4 originally derived from a field strain of ALV J termed R5-4 (Lupiani *et al.*, 2003) were used in this study. ADOL R5-4 was shown to have similar biological characteristics as ADOL Hc1 (Lupiani *et al.*, 2003). Viruses were propagated and titrated in ADOL Line 0 chicken embryo fibroblasts that are known to be resistant to subgroup E ALV(C/E).

Virological and serological assays. *Virus isolation.* Plasma samples collected during each sampling period were frozen at –70°C until they were tested for viraemia by virus isolation, which was done according to the procedures described earlier (Fadly & Witter, 1998; Pandiri *et al.*, 2007). Briefly, about 100 µl undiluted plasma was added to 0.18×10^6 chicken embryo fibroblasts suspended in 4% calf serum Leibowitz's L-15-McCoy's 5A tissue culture medium (1:1) (LM) containing penicillin, streptomycin, amphotericin B and 0.004 IU heparin in 24-well tissue culture plates. Uninoculated cells in tissue culture media were used as negative control. On the following day, the 4% calf serum LM media was replaced with 1% calf serum LM media. The plates were incubated at 37°C and 4 % CO_2 for 7 to 9 days before the cells were completely lysed with 50 µl of 0.5% Tween 80 (Sigma Chemical Co., St Louis, Missouri, USA) and two alternate cycles of freezing at −70°C and thawing at 37°C. About 100 µl cell lysate was used to test for p27 ALV group-specific antigen (GSA) by enzyme-linked immunosorbent assay (Smith *et al.*, 1979). The p27 GSA enzyme-linked immunosorbent assay was carried out using rabbit anti-p27 polyclonal antibody-coated immunolon® plates (Dynatech, Chantilly, Virginia, USA), rabbit anti-p27 antibody conjugated to horseradish peroxidase (SPAFAS, Storrs, Connecticut, USA) and TMB substrate (3,3′,5,5′-tetramethyl benzidine) (BD Biosciences Pharmingen, San Diego, California, USA). The plate was read at an absorbance of 630 nm using a MRX microplate reader (Dynex, Chantilly, Virginia, USA).

Virus micro-neutralization. Plasma samples were tested for neutralizing antibody against ALV J viral stocks that were used to infect the experimental chickens. Virus micro-neutralization assays were performed as described earlier (Fadly & Witter, 1998). In précis, the plasma samples were diluted 1:5 in serum-free LM media and incubated at 56°C for 30 min to denature the complement factors. About 500 to 1000 ALV J viral particles in 50 µl LM media are incubated with 50 µl heat-denatured 1:5 diluted plasma in 96-well flat-bottomed tissue culture plates for 45 min at 37°C and 4 % CO_2. After the incubation, about 1×10^5 cells in 150 µl of 4 % calf serum LM media were pipetted into each of the 96 wells and incubated at 37°C and 4% CO_2 for 7 to 9 days. At the end of incubation, the cell cultures were completely lysed with 20 µl of 0.5 % Tween 80 (Sigma Chemical Co.) and were subjected to two alternate cycles of freezing at −70°C and thawing at 37°C. The cell lysates were tested for p27 GSA enzyme-linked immunosorbent assay as described earlier. Any sample that had a reading of one or was negative for the p27 GSA was considered positive for NAb against ALV J, and *vice versa*.

Pathology. All experimentally inoculated chickens were necropsied, and diagnosis of tumours was made by gross and microscopic examinations of affected tissues. For microscopic examination, tissues were fixed in 10% neutral buffered formalin, processed, sectioned and stained with haematoxylin and eosin.

Polymerase chain reaction. Genomic DNA was extracted from different tissues using the Puregene™ DNA isolation kit (Gentra System Inc., Minneapolis, Minnesota, USA), and was amplified with various ALV J oligonucleotide primers. The PCR was conducted using four separate

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Table 1. *Selection of chickens based on the ALV J infection profile*[a]

Infection profile[b]	Chicken type	Number of chickens	Treatment			
			Virus strain	Median tissue culture infectious dose	Inoculation route	Age
ntV+A−	Meat-type	5	ADOL R5-4	10 000	Intra abdominal	1 day
V+A+	Meat-type	8	ADOL R5-4	10 000	Intra abdominal	1 day
V−A+	Meat-type	5	ADOL R5-4	Exposed by contact at 1 day of age		
	Line 0	5	ADOL R5-4	10 000	Intra abdominal	1 day
V−A−	Meat-type	1	ADOL R5-4	10 000	Intra abdominal	1 day
	Line 0	1	ADOL R5-4	10 000	Intra abdominal	1 day
tV+A−	Line 0	1	ADOL Hc1	10 000	*In ovo* (yolk sac)	5 ED

[a]In this study, 26 chickens were selected retrospectively from two previous experiments based on the infection profile.
[b]The different infection profiles are based on viraemia as tested by virus isolation and on NAb as tested by virus microneutralization assay on samples collected at 8, 12, 16, 20, 24 and 32 weeks post hatch. The different infection profiles are defined in Materials and Methods.

oligonucleotide primers pairs—6J/Smith2 and F5/Smith2 (Smith *et al.*, 1998a; Silva *et al.*, 2000), H5/R11 (Silva *et al.*, 2000), H5/H7 (Smith *et al.*, 1998b)—amplifying the *env* gene of ALV J provirus. The sequence of the oligonucleotide primer pairs were as follows: 6J, 5′-CTT GCT GCC ATC GAG AGG TTA CT-3′; F5, 5′-GGT ATT TTC TTG ATT TGT GGG G-3′; Smith2, 5′-AGT TGT CAG GGA ATC GAC-3′; H5, 5′-GGA TGA GGT GAC TAA GAA AG-3′; R11, 5′-TGG GGG TGG GAA GGG AGG GT-3′; and H7, 5′-CGA ACC AAA GGT AAC ACA CG-3′. Each reaction had a final volume of 25 µl and included 50 ng DNA, and the master mix consisted of 3.5 µl 10 × PCR buffer (10 mM Tris–HCl (pH 8.3 at 25°C), 500 mM KCl, and 15 mM $MgCl_2$, 1.5 µl (12.5 pM) forward primer (6J, F5, H5), 1.5 µl (12.5 pM) reverse primer (Smith2, R11), 0.2 µl (100 mM) dNTPs, 0.125 µl Taq polymerase, and 18.2 µl water. The PCR conditions for 6J/Smith2 or F5/Smith 2 oligonucleotide primer sets were 95°C for 3 min, 95°C for 1 min, 57°C for 1 min, 72°C for 2 min, go to step 2 for 29 times, 72°C for 5 min, and 4°C hold. The PCR conditions for oligonucleotide primers H5/R11 were similar to 6J-Smith2 or F5-Smith2 except for a lower extension time of 30 sec. A 'touch down' PCR was performed using oligonucleotide primers H5/H7 following the published protocols (Smith *et al.*, 1998b). The PCR amplified products were run on a 1% agarose gel. The product sizes for 6J/Smith2 (upstream of the coding region of gp85 to LTR), F5/Smith2 (within the coding region of gp85 to LTR), H5/R11 (upstream of the pol region to gp85) and H5/H7 (upstream of the pol region to gp85) are 2.3 kb, 1.5 kb, 445 b, and 545 b, respectively. The positive controls included DNA from tV+A− chickens and myelocytoma tumour tissue. The negative controls included DNA from unexposed meat-type and ADOL Line 0 chickens, as well as various stock DNA samples from other uninfected meat-type chickens. Samples with negative PCR results were tested with GAPDH primers (GAPDH-TM.5, 5′-GGAGTCAACGGATTTGGCC-3′; and GAPDH-TM.3, 5′-TTTGCCAGAGAGGACGGC-3′) as internal control (Silva *et al.*, 2007).

Immunohistochemistry. A modified avidin–biotin peroxidase complex method (Hsu *et al.*, 1981) using the Vectastain® ABC kit (Vector Laboratories; Burlingame, California, USA) was performed. Monoclonal antibody G2-3 generated from ADOL Hc1 (Qin *et al.*, 2001) that is specific for the gp85 protein of ALV J was used at a working dilution of 1:500. The positive control included tissues from tV+A− chickens and the negative controls included tissues from unexposed chickens as well as slides incubated with phosphate-buffered saline instead of primary antibody.

Scoring of tissues. The slides were read without the knowledge of infection profile to avoid bias. The gp85 staining was subjectively scored: 0 = no positive cells, 1 = a few scattered positive cells, 2 = moderate number of positive cells, and 3 = large number of positive cells. Mean tissue scores were calculated for each tissue within each group of chickens.

Results

Distribution of gp85 in various tissues from chickens with different ALV J infection profiles. Expression of gp85 viral antigen in various tissues (adrenal gland, bone marrow, gonad, heart, kidney, liver, lung, pancreas, proventriculus, sciatic nerve, spleen, and thymus) from chickens with four different ALV J infection profiles is summarized in Table 2. In ntV+A− and V+A+ chickens, the expression of gp85 was found in all tissues tested with the exception of the sciatic nerve. There were no significant differences in the frequency or the mean tissue scores between the ntV+A− (1.2 to 3.0) and V+A+ (1.3 to 2.3) groups. However, the Line 0 tolerant viraemic chicken (tV+A−) showed a high level of gp85 expression in all tissues (3.0), with the exception of the sciatic nerve that had a score of 1.0. In addition, the lymphocytes in the thymus, and spleen, as well as lymphoid aggregates within the proventriculus, kidney and liver, stained positive for gp85 in the tV+A− chicken (Figure 1). In contrast to results obtained from viraemic chickens, no expression of gp85 was observed in V−A+ chickens. The V−A− meat-type and ADOL Line 0 chickens did not exhibit any gp85 expression within all the tissues examined.

Several chickens with different ALV J infection profiles developed tumours; namely, nephroblastoma (one V+A+ chicken), myelocytoma (two V+A+ chickens and one tV+A− chicken) and hemangiopericytoma (one ntV+A− chicken). In all cases, tumour cells demonstrated a very strong gp85 expression (3.0) (data not shown).

Distribution of proviral DNA in various tissues from chickens with four different infection profiles. The results of testing various tissues for the presence of ALV J provirus (using primer sets H5/H7) in the genomic DNA from meat-type and ADOL Line 0 chickens classed as tV+A−, ntV+A−, V+A+, V−A+ and V−A− are also summarized in Table 2. Primer sets 6J/Smith2 and F5/Smith2 amplifying *env* sequences yielded almost identical PCR results and consistently demonstrated ALV J proviral DNA in all the tissues collected from viraemic chickens (tV+A−, ntV+A−, and V+A+) (data not shown). Further, there were no differences in tissue distribution of ALV J provirus in any of the tissues tested from viraemic chickens (tV+A−, ntV+A−, and V+A+), regardless of the line of chickens used. In contrast, no ALV J proviral DNA was detected in any of

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Table 2. *Detection of ALV J proviral DNA and gp85 antigen in various tissues from meat-type and Line 0 chickens with different infectious status*[a]

Line/infection class[b]	Number of chickens	Test	Adrenal gland[c]	Bone marrow	Gonad	Heart	Kidney	Liver	Lung	Pancreas	Proventriculus	Sciatic nerve	Spleen	Thymus
Meat-type, ntV+A−	5	PCR	5/5[c]	5/5	3/3	4/4	5/5	5/5	3/3	5/5	5/5	2/4	3/3	5/5
		IHC	5/5[c]	2/5	3/5	5/5	5/5	4/5	3/5	5/5	5/5	0/5	5/5	5/5
			2.4[d]	1.5	1.3	1.8	2.2	1.0	2.0	2.4	3.0	0	1.4	1.2
Meat-type, V+A+	8	PCR	5/6	6/7	6/6	8/8	8/8	7/8	6/7	8/8	8/8	6/7	6/7	6/7
		IHC	7/7	5/7	7/8	5/8	8/8	7/8	7/8	8/8	8/8	0/8	8/8	6/8
			1.9	2.2	1.6	1.5	2.3	1.6	1.4	2.0	2.0	0	1.8	1.3
Meat-type, V−A+	5	PCR	3/4	1/4	0/4	1/4	0/4	0/4	1/4	2/4	2/4	2/4	2/4	1/3
		IHC	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
			0	0	0	0	0	0	0	0	0	0	0	0
Line 0, V−A+	5	PCR	2/5	2/4	1/5	5/5	4/5	2/5	4/5	3/5	2/5	0/5	4/5	2/4
		IHC	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
			0	0	0	0	0	0	0	0	0	0	0	0
Meat-type, V−A−	1	PCR	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
		IHC	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
			0	0	0	0	0	0	0	0	0	0	0	0
Line 0, V-A−	1	PCR	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
		IHC	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
			0	0	0	0	0	0	0	0	0	0	0	0
Line 0, tV+A−	1	PCR	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
		IHC	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
			3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	1.0	3.0	3.0

[a]Proviral DNA detected by PCR using primer set H5/H7 and gp85 detected by IHC using G2-3 monoclonal antibody.
[b]The viraemia and NAb data from the meat-type and ADOL Line 0 chickens are classified as defined in Materials and Methods.
[c]The discrepancy in the number of chickens and some of the respective samples was due to lack of the specific tissue for testing.
[d]Mean subjective tissues scores. The lowest is 0 and the maximum is 3.0.

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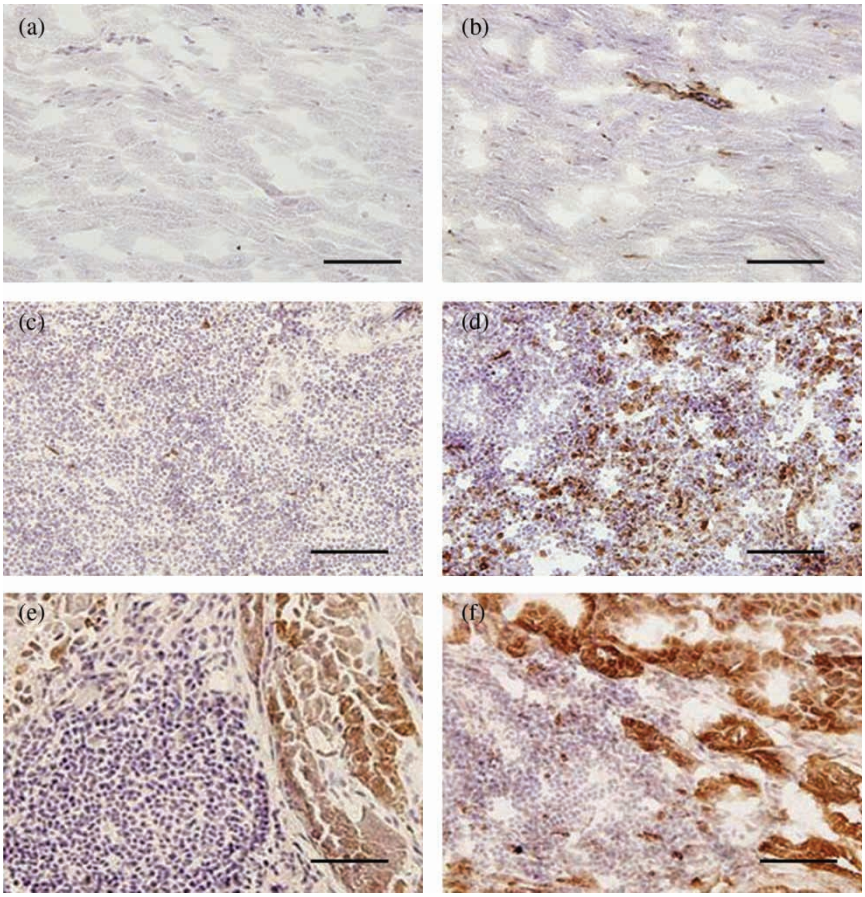

Figure 1. *Distribution of ALV J gp85 in various tissues from 32-week-old chickens categorized as ntV+A− (1a, 1c, 1e), and tV+A− (1b, 1d, 1f). 1a: Sciatic nerve section from a ntV+A− meat-type chicken with no gp85 expression. Bar = 120 μM. 1b: Sciatic nerve section from a tV+A− ADOL Line 0 chicken with gp85 expression in endothelial cells, and nerve fibres. Bar = 120 μM. 1c: Spleen from a ntV+A− meat-type chicken with scattered reticular cells with gp85 expression. Bar = 120 μM. 1d: Spleen from a tV+A− ADOL Line 0 chicken with gp85 expression in macrophages, endothelial cells, and lymphocytes. Bar = 120 μM. 1e: Proventriculus from a ntV+A− meat-type chicken with strong gp85 expression in the glandular epithelium but no staining in lymphoid aggregates. Bar = 95 μM. 1f: Proventriculus from a tV+A− ADOL Line 0 chicken with strong gp85 expression in the glandular epithelium and within lymphoid aggregates. Bar = 95 μM*

the tissues collected from V−A+ meat-type or Line 0 chickens using primer sets 6J/Smith2 and F5/Smith2. Results obtained using H5/R11 or H5/H7 primer sets amplifying *env* sequences yielded similar results for tissues collected from viraemic tV+A−, ntV+A−, and V+A+ meat-type or ADOL Line 0 chickens (data not shown). Using H5/R11 PCR, results for tissues from V−A+ meat-type or Line 0 chickens were not clear due to spurious amplification, and no conclusive results could be obtained despite performing a PCR under 'touch down' conditions (Don *et al.*, 1991). However, using H5/H7 oligonucleotide primers on tissues collected from V−A+ meat-type or Line 0 chickens, PCR results were clear with no spurious amplification although the amplified PCR product was faint. The PCR amplified product in viraemic chickens (V+A−, ntV+A− and V+A+) yielded a bright band upon ultraviolet transillumination, unlike the PCR-amplified product from seroconverted chickens (V−A+) that yielded a faint band. The pattern of tissue distribution of ALV J proviral DNA in V−A+ meat-type chickens differed from that of Line 0 chickens. Tissues from V−A+ Line 0 chickens demonstrated proviral DNA more frequently than V−A+ meat-type chickens. All the tumour tissues tested in the study were positive for ALV J proviral DNA with any of the above primer sets (data not presented). All of the primer sets failed to demonstrate the presence of ALV J provirus within the tissues examined from V−A− meat-type and ADOL Line 0 chickens.

Discussion

Results from this study demonstrate that the distribution of gp85 was directly correlated to the viraemia status of the chicken, regardless of the NAb status. High levels of gp85 expression were found in viraemic chickens (tV+A−, ntV+A− and V+A+), but not in non-viraemic seroconverted chickens (V−A+). The data also show that using primer set H5/H7, but not sets 6J/Smith2, F5/Smith2 or H5/R11, proviral DNA was detected in tissues collected from non-viraemic seroconverted chickens. The tissue distribution of ALV J antigen in viraemic chickens was in agreement with previous studies (Dougherty & Di Stefano, 1967; Arshad *et al.*, 1997; Gharaibeh *et al.*, 2001; Stedman *et al.*, 2001; Williams *et al.*, 2004). All previously reported studies used tissues from viraemic chickens that had extensive distribution of gp85, similar to tV+A−, ntV+A− and V+A+ chickens used in the present study. In addition, data from the present study show that gp85 and ALV J proviral distribution was similar in viraemic chickens with (V+A+) or without NAbs (ntV+A−).

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Minor differences were found in the pattern of gp85 expression between tV+A− and non-tolerized viraemic chickens (ntV+A−, V+A+). The tV+A− chicken had positive staining in the nerve fibres and endothelial cells in the sciatic nerve, and lymphoid aggregates in several tissues. This difference in pattern of distribution of gp85 is probably due to high viraemic load found because of a total lack of immune response against ALV J in tolerized chickens, or it might be due to differences in viral strain used to inoculate the tV+A− chicken in the present study. The latter explanation is unlikely, as ADOL R5-4 and ADOL Hc1 have been shown to have very similar biological properties and the monoclonal antibody G2-3 detects both the viruses (Lupiani *et al.*, 2003). Also, gp85 expression was consistently detected in the sciatic nerve and lymphocytes as in other studies involving tolerized chickens that had been inoculated with different ALV J strains (Arshad *et al.*, 1997; Stedman *et al.*, 2001; Williams *et al.*, 2004).

The presence of NAb did not influence the gp85 or the proviral DNA distribution in viraemic meat-type chickens (V+A+). The V+A+ category was included only for meat-type chickens since this infection pattern is very common in this type of chickens but occurs very rarely in Line 0 chickens (Witter *et al.*, 2000; Mays *et al.*, 2005; Pandiri, 2005). Previous data from ALV J infection in Line 0 and meat-type chickens has always demonstrated the ability of Line 0 chickens to clear viraemia with an efficient NAb response better than meat-type chickens (Mays *et al.*, 2005). The high incidence of V+A+ in meat-type chickens following infection at hatch may be due to the emergence of ALV J NAb escape variants that were not neutralized by the circulating antibodies against the inoculated strain of virus (Pandiri, 2005). The exact role of the immune system and the chicken's endogenous virus status in the development of this high incidence of V+A+ in meat-type chickens need to be determined.

Our previous studies demonstrated that ALV J seroconverted chickens could revert to viraemia when subjected to ACTH-induced stress (Pandiri, 2005) One of the main objectives of the current study was to elucidate the distribution of virus in chickens that have successfully cleared ALV J viraemia with a consistent NAb response (V−A+). In this study, we have demonstrated that the tissue distribution of proviral DNA in V−A+ meat-type and ADOL Line 0 chickens is very similar to proviral DNA distribution in viraemic chickens of either line. However, the frequency of tissue distribution of proviral DNA is lower than viraemic chickens. Sensitivity of the test seems to be critical for detecting ALV J provirus in V−A+ chickens since only one primer set (H5/H7) was able to demonstrate ALV J provirus in V−A+ chickens. As more sensitive tests are developed, the frequency of ALV J provirus in V−A+ chickens may be comparable with that of viraemic chickens.

Infection by retroviruses like ALV J involves reverse transcription and genomic integration of the virus in proviral DNA form. In this study, proviral DNA sequences but not gp85 expression was observed in seroconverted non-viraemic chickens (V−A+), implying a latent ALV J infection in these chickens. Apparently, under certain conditions, ALV J seroconverted non-viraemic chickens are able to revert to viraemia (Pandiri, 2005). This reversion to viraemia may be due to switching from latent to productive infection that is characterized by transcription of proviral DNA into viral mRNA, and consequently into an infectious viral particle (Ahmed *et al.*, 1997). The factors responsible for switching the latent infection into a productive infection need to be determined.

High expression of viral antigen (gp85) was also noted in ALV J-induced tumours such as myelocytomas and nephroblastomas (data not shown). This finding is in contrast to previous work by Arshad *et al.* (1999), who reported that Gag protein or viral transcripts could not be demonstrated by immunohistochemistry or *in-situ* hybridization, respectively, in 966-virus-transformed myelocytoma, although virus budding could be demonstrated by electron microscopy. This may be due to the following reasons: sensitivity of the techniques used; formalin or Bouin's fixed tissues embedded in paraffin (instead of cryosections); possible genetic defectiveness of strain 966, an acutely transforming virus ALV J virus; or individual variation in viral antigen and proviral levels in retrovirus-induced tumour. It has been demonstrated that neither viral replication nor high levels of gene expression are necessary for retrovirus-induced lymphoma development (Neel *et al.*, 1981; Payne *et al.*, 1981). Thus, this may also be true for the differences in viral antigen and/or proviral levels in different ALV J-induced myelocytomas.

Finally, to our knowledge, this is the first study that demonstrated tissue-specific expression of ALV J antigen as well as proviral DNA in chickens classified into five different infection profiles. Our results demonstrate a direct correlation between viraemia and tissue distribution of gp85, regardless of the NAb response and strain of chickens; and demonstrate that, using certain primer sets, ALV J provirus could be detected in seroconverted viraemia-free chickens.

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